Dexamethasone blocks antioestrogen- and oxidant-induced death of pituitary tumour cells

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Abstract

The oestrogen receptor is fundamental to the growth and survival of the rat pituitary tumour cell line, GH_3. Our previous studies have shown that antioestrogens such as RU 58668 and ZM 182780 will reduce the rate of cell division and also induce cell death. Death of these cells in response to antioestrogen treatment appears to be due to a heightened sensitivity to reactive oxygen species (ROS). As part of a study to determine the cross-talk between steroid receptor systems in these cells, we have observed that the glucocorticoid, dexamethasone (Dex), inhibits antioestrogen-induced cell death. Cell death induced by H_2O_2 is enhanced by ZM 182780 and this effect is also blocked by Dex. As apoptotic cell death in a number of systems involves an early loss of mitochondrial membrane potential ($\Delta\Psi_m$), we have performed detailed studies on the time-course of $\Delta\Psi_m$ loss in relation to the loss in cell membrane function. These studies have indicated that a loss of $\Delta\Psi_m$ parallels a loss of cell membrane function – this is more characteristic of necrosis than of apoptosis. From microscopic observations of these cells in response to H_2O_2, it has been noted that early cell membrane blebbing, induced by H_2O_2, is blocked in the presence of ZM 182780. Cell membrane blebbing can precede necrosis as well as apoptosis and it is thought to involve cytoskeletal changes, for which localised glycolytic reactions provide ATP. These observations, together with those showing that removal of glucose, but not inhibition of mitochondrial function, enhances ROS-induced cell death, prompted studies on the glycolytic pathway. As a strong candidate mechanism, it would appear that, via an effect on one of the rate-limiting glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase, Dex is able to overcome the antioestrogen-enhanced loss of glycolytic function following exposure of cells to ROS. This report contributes to the growing body of evidence showing that glucocorticoids provide a survival advantage to both normal and tumour cell types.

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Introduction

A characteristic response of tumour cell types that contain the oestrogen receptor (ER) is that oestrogens induce growth (Katzenellenbogen *et al.* 1979). There are exceptions to this rule, however. Although a considerable amount of evidence has accumulated that prolactin-secreting human pituitary tumours contain the ER (Friend *et al.* 1994), there is little strong evidence to indicate that these tumours are oestrogen-induced or even oestrogen dependent for growth. In contrast, this is not the case for the rat. Several strains of rat develop oestrogen-induced prolactin-secreting tumours (Wiklund *et al.* 1981). An ER-containing rat pituitary cell often used for studies on the effect of estrogens is the GH_3 cell line (Tashjian *et al.* 1968). Although these cells were not originally obtained from an oestrogen-induced tumour, both growth and prolactin secretion are suppressed by the addition of antioestrogens to cells in culture (Newton *et al.* 1994*a*). Despite these observations with antioestrogens, a peculiarity of these cells is that growth is not always responsive to oestrogens (Haug & Gautvik 1976, Sorrentino *et al.* 1976). A few years ago this problem was solved by studies using the pure antioestrogen, ZM 182780, where it was shown that the ER within these cells functions as a transcription factor in the absence of oestrogens (Newton *et al.* 1994*b*). It was apparent from these studies that the receptor could be made transcriptionally active by estrogens and also in response to signalling pathways activated by the growth factors, insulin, insulin-like growth factor-I (Newton *et al.* 1994*c*) and the cytokine, interleukin-2 (Newton *et al.* 1994*a*).

More recent work with this cell line has demonstrated that extended culture in the presence of the pure steroidal antioestrogen, ZM 182780, induces cell death, an effect that is fully reversed by oestrogens (Newton 1995). The

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mechanism for this has been partially elucidated by studies showing that cell death is due to the accumulation of reactive oxygen species (ROS), and that sensitivity to these agents is enhanced by antioestrogen-induced loss of ER function (Newton *et al.* 1999). A major conclusion from these studies was that functional ablation of the ER sensitises cells to ROS-induced metabolic collapse, leading to cell death with some of the characteristics of apoptosis (Schwartzman & Cidlowski 1993).

In addition to the ER, the other members of the superfamily of nuclear receptors (Hurd & Moudgil 1998) known to exist within the GH_3 cells are the progesterone receptor (Newton *et al.* 1994*a*), the glucocorticoid receptor (Perrone *et al.* 1980), the thyroid hormone receptor and the retinoic acid receptors (Morita *et al.* 1990). Studies over the past decade have demonstrated marked functional cross-talk between receptor systems (Bunone *et al.* 1996). In this respect, tri-iodothyronine (T_3)-induced growth of GH_3 cells has been shown to be inhibited by antioestrogens (Zhou-Li *et al.* 1992). Similarly, the synthetic glucocorticoid, dexamethasone (Dex), has been shown to inhibit both oestrogen- and T_3-induced growth of GH_3 cells (Zhou-Li *et al.* 1991). Indeed, it would appear that Dex is able to reduce oestrogen-induced growth in other systems (Bigsby & Young 1993). During our experiments on the functional cross-talk between nuclear receptor systems in GH_3 cells, one of the combinations tested was Dex and the pure antioestrogen, ZM 182780 (Wakeling & Bowler 1991). Cell death induced by exposure to antioestrogen for several days was completely blocked by Dex. In the current report, we have determined whether this response was specific for the interaction of Dex with the glucocorticoid receptor or whether it is shared by other steroid hormones. Based on our previous observations, outlined above, we have also tested whether Dex blocks cell death due to ROS generated as the result of the addition of the pro-oxidant, H_2O_2. Finally, by modulating glycolytic function, we have determined the role that the cellular bioenergetics status plays in the response of these cells to both the antioestrogen and to the glucocorticoid, Dex.

Materials and Methods

Reagents

All reagents for cell culture were obtained from Life Technologies (Paisley, Strathclyde, UK). The cell death ELISA was obtained from Roche Diagnostics Ltd (Lewes, E Sussex, UK). Fluorescent probes for flow cytometry were obtained from Molecular Probes (Cambridge Bioscience, Cambridge, Cambs, UK). Other flow cytometry reagents were obtained from Beckton Dickinson UK Ltd, Oxford, UK. All other reagents were obtained from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK). The pure antioestrogen was obtained as a gift from Zeneca Pharmaceutucals Ltd (Macclesfield, Cheshire, UK).

Cell culture

Pituitary GH_3 cells as previously described (Newton *et al.* 1994*b*) were maintained in Dulbecco's modified Eagle's medium (DMEM) with phenol red, penicillin (50 U/ml) streptomycin (50 μg/ml), amphoteracin (2 μg/ml) and 10% foetal calf serum (Myoclone; Gibco, Paisley, Strathclyde, UK). For the majority of studies reported here, experiments were conducted on cells seeded onto 48-well culture plates at densities over the range 4000 to 10 000 cells per cm^2. Cells were kept at 37 °C in an atmosphere of 5% CO_2 in air. For flow cytometry and enzyme measurements, cells were harvested from 75 cm^2 culture flasks by the addition of trypsin/EDTA and resuspended in culture medium. For flow cytometry, cells were treated in suspension at 37 °C in a gas atmosphere of 5% CO_2 in air.

Transfection

Plasmids for transfection were the MMTV-Luc and ΔMTV-Luc as previously described (Newton *et al.* 1994*c*). After seeding and cell attachment, media were changed to phenol red-free DMEM containing 10% charcoal-stripped serum and 24 h later transfections were made with 1 μg plasmid DNA and the reagent FUGENE, according to the manufacturer's instructions (Roche Diagnostics Ltd). After a further 24 h, luciferase activity was measured in the cell lysates using the Promega luciferase assay system (Madison, WI, USA). All values obtained as relative light units, measured with a luminometer, were expressed as fold control (no treatment).

Determination of cell numbers and cell death

We have previously shown that viable cell numbers can be quantitated by the addition of the diazo dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazdium bromide (MTT) to cells for 4 h to give final concentration of 0·1 mg/ml (Newton 1995). The blue crystals formed were solubilised by the addition of sodium dodecylsulphate in HCl to give final concentrations of 10% and 10 mM respectively. Absorbance values were determined at 550 nm. For the experiment shown in Fig. 3, a direct estimate of cell numbers was made using a Coulter Counter (Beckman Coulter Ltd, High Wycombe, UK). Prior to assay, cell monolayers were washed twice with phosphate-buffered saline (PBS) to remove poorly attaching, dead cells.

As described previously (Newton *et al.* 1999), a quantitative measure of cell death was made by determining DNA fragmentation using the ELISA^{PLUS} assay (Roche Diagnostics Ltd).

Fluorescence microscopy

Normal light (Image Modulated Contrast) and fluorescence microscopy were performed with a Leica DMIL

fitted with a SPOT^{JUNIOR} digital camera (Diagnostic Instruments, Oxnard, CA, USA). Photo-quality pictures were produced with a Hewlett Packard 970 Cxi. To determine the integrity of cell membranes, a mixture of propidium iodide (PI) and Hoechst 33342 was added to the cell culture medium to give final concentrations of 10 μg/ml and 1μg/ml respectively. After 5 min at 37 °C, cells were visualised following exposure to normal and UV light (380 nm). Cells with disrupted membranes preferentially gave red nuclear fluorescence due to the uptake of PI. Cells with intact membranes gave blue nuclear fluorescence, due to the uptake of the cell-permeable Hoechst 33342 fluorochrome.

Flow cytometry for changes in mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by a previously published method (Decaudin *et al.* 1997). Control and steroid pretreated (24 h) cells were harvested from 75 cm^2 flasks by the addition of trypsin/EDTA and exposed, in suspension, to 150 μM H_2O_2. After 1 h and thereafter at approximately 30-min intervals, 300 μl cell suspension was taken to which was added 30 μl PBS containing the cationic dye, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes; final concentration 80 μM), and PI (1 μg/ml). Labelled cells were then incubated for 10 min at 37 °C prior to sampling with a FACS^{Calibur} flow cytometer (Becton Dickinson UK Ltd) using an argon laser tuned to 480 nm. Fluorescence emissions were detected in green and red channels and plotted on the x axis against the number of cell on the y axis. Loss of $\Delta\Psi_m$ was detected as a decrease in green fluorescence intensity. Loss of membrane integrity was observed by an increase in the red fluorescence.

In addition to the flow cytometry method, the $\Delta\Psi_m$ was also determined by fluorescence microscopy using the cationic dye, 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidolycarbocyanine iodide (JC-1) and exposure to light at 480 nm. As a cation, this dye is taken-up by mitochondria according to the membrane potential (Coulter *et al.* 2000). At low potential, the dye exists as a monomer and fluoresces green. At high potential, JC-1 forms 'J' aggregates and fluoresces red.

Cellular ATP concentrations and glyceraldehyde-3-phosphate dehydrogenase activity

ATP was determined in cell lysates with a luminescent assay. Medium was removed from treated cells in 48-well plates and 0·5 ml distilled water was added. After a period of 1–2 min the cell lysate was aspirated and boiled for 5 min. This was then diluted with a further 1 ml distilled water and 100 μl aliquots were mixed with a 100 μl aliquot of a luciferase/luciferin mixture provided by Sigma-Aldrich Co. (FL-AA). Values were recorded as relative light units and are expressed as a percentage of those obtained for untreated control cells. Values obtained from the experiments fell within the wide linear range of the assay.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was determined as described by Halder *et al.* (1993). In brief, cells harvested from 75 cm^2 flasks were sonicated on ice in PBS to break cytosolic membranes. The sonicate was then centrifuged at 1500 ***g*** and an aliquot of the crude cytosol containing supernatant was taken for spectrophotometric GAPDH assay according to the methodology described by Scheek & Slater (1982). Values obtained for enzyme activity in μmol NADH produced/min were corrected for protein content and then expressed as percentage of those obtained for untreated, control cells.

Statistics

Where indicated, statistical analysis was performed on a minimum of three replicates using the Student's *t*-test. With the exception of Fig. 5, figures are reproduced as representative experiments from at least five identical studies. All values given in the figures are expressed as means ± S.D. For clarity, error bars have been omitted from some figures.

Results

Functional activation of steroid receptors and effect of ligands on ZM 182780-induced cell death

To determine the functional activity of the steroid receptors present in GH_3 cells, transfection was performed with the MMTV-Luc and ΔMTV-ERE-Luc reporter plasmids (Newton *et al.* 1994*b*) followed by a 24-h treatment period with the progestin, promogestone (R5020, 10 nM), the androgen, dihydrotestosterone (DHT, 10 nM), the glucocorticoid, Dex (100 nM) and the oestrogen, oestradiol (E_2, 1 nM). To ensure the removal of endogenous steroids, cells were maintained prior to treatment in phenol red-free medium containing charcoal-stripped serum. Figure 1 shows that all steroids induced reporter gene expression and that the respective receptor antagonists, ZK 98299 (progesterone receptor (PR), 10 μM), RU 486 (glucocorticoid receptor (GR), 10 μM), hydroxyflutamide (androgen receptor (AR), 10 μM) and ZM 182780 (ER, 100 nM) blocked transcription of the reporter genes. It should be noted that for transfections peformed with the ΔMTV-ERE-Luc plasmid, the antioestrogen reduced luciferase expression below that of the oestrogen-free control. This is consistent with earlier studies showing that the ER within these cells is transcriptionally active in the absence of oestrogen (Newton *et al.* 1994*c*).

To determine whether the ligand-activated receptors altered the response of cells to the antioestrogen, cultures

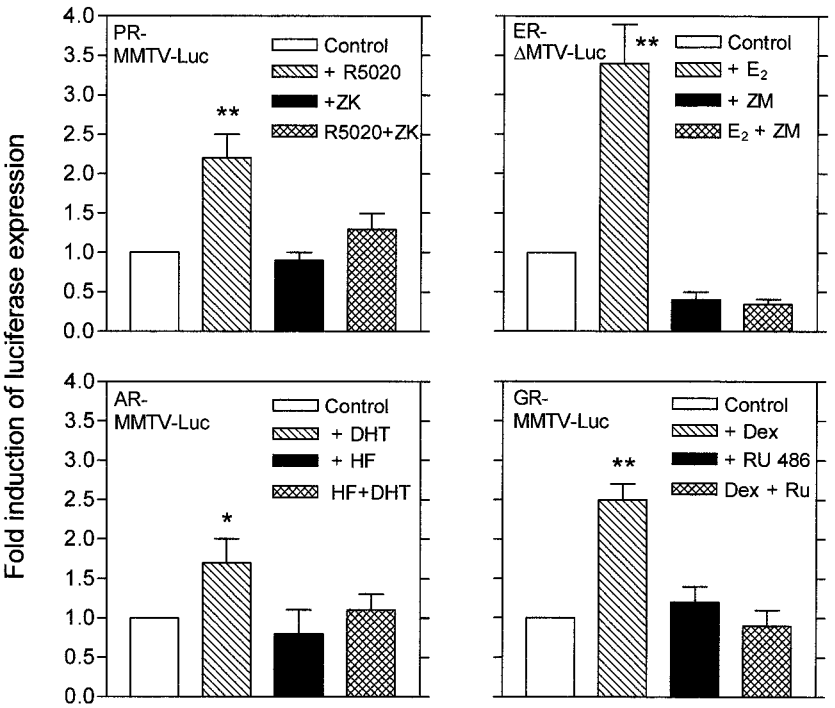

Figure 1 Steroid receptor activation in GH_3 cells. Cells were transfected with 1 μg MMTV-Luc or ΔMTV-ERE-Luc plasmids and then exposed to steroids in the absence and presence of specific receptor antagonists. Luciferase activity detected 24 h after transfection is expressed as a function of that detected in transfected but otherwise untreated cells. Steroid/steroid antagonist concentrations: R5020, 10 nM; ZK 98299 (ZK), 10 μM; E_2, 1 nM; ZM 182780 (ZM), 100 nM; DHT, 10 nM; hydroxyflutamide (HF), 10 μM; Dex, 100 nM; RU 486 (Ru), 10 μM. Error bars in this and other figures are means ± S.D. *P<0·01, **P<0·001 versus control.

were maintained in the presence of 100 nM ZM 182780 and a dose range of Dex, R5020, DHT, oestrone (E_1) and E_2. Figure 2 demonstrates the response of GH_3 cells measured as metabolic activity by the addition of the redox sensitive diazo dye, MTT. In comparison to E_1 and E_2, where metabolic activity was restored to that of cells untreated with antioestrogen, only Dex was observed to partially reverse the antioestrogen-induced decrease of metabolic activity. For estimates of the proportion of viable cells, determined by the addition of the fluorochromes, Hoechst 33342 and PI, Fig. 2b shows that Dex completely blocked the antioestrogen-induced loss of cell viability. It should be mentioned that unlike E_2, E_1 was able to maintain cell viability at concentrations 100-fold lower than for E_2. As E_1 has a lower affinity for the ER than E_2, this suggests that either the effect of E_1 is mediated via an ER isoform to which the antioestrogen fails to bind, or that the effect of E_1 is mediated via an ER-independent pathway.

To provide confirmation of the effect of Dex, a further experiment was performed over a time-course of 9 days, where cell numbers were measured using a Coulter Counter. Figure 3 shows that in the presence of ZM 182780 alone, cell numbers fell by day 5, but growth continued at a low rate in the combined presence of 100 nM ZM 182780 and 100 nM Dex.

Antioestrogen- and exogenous oxidant-induced cell death is blocked by Dex

We have previously shown that death induced by ZM 182780 results in extensive DNA fragmentation (Newton *et al.* 1999). Using the same ELISA method, we determined the effect of Dex on the time-course of antioestrogen-induced DNA fragmentation. Figure 4a shows that over a time-course of 7 days, DNA fragmentation began to rise from day 4 and this was completely blocked by 100 nM Dex. Given that we have previously shown that oxidants are involved in antioestrogen-induced death of GH_3 cells, we next tested the response of cells to the pro-oxidant, H_2O_2. Previously untreated control cells and cells exposed to 100 nM ZM 182780 for 48 h were treated with a dose range of H_2O_2 for 2 h. At concentrations of H_2O_2 around 100–200 μM, DNA fragmentation was markedly enhanced for antioestrogen-exposed cells (Fig. 4b). As indicated previously (Newton

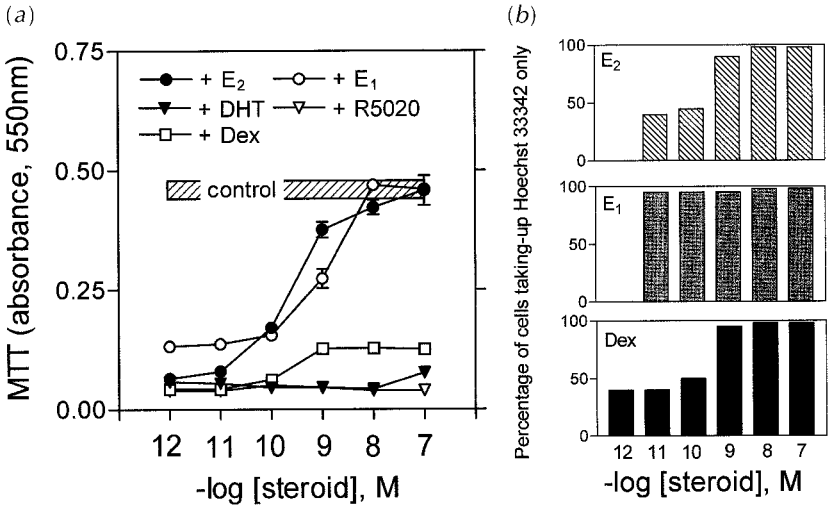

Figure 2 Response of antioestrogen-treated cells to a dose range of steroids measured in (*a*) as metabolic activity after 5 days of treatment and in (*b*) as the proportion of viable cells. Apart from metabolic activity of control cells, represented as the hatched bar (means ± S.D.) in (a), all other cells received 100 nM ZM 182780 along with the steroids at the concentrations indicated.

et al. 1999), over a time-period of 1–2 h, GH_3 cells paradoxically fail to show DNA fragmentation at the higher pro-oxidant doses. Figure 4b also shows that pretreatment of cells with 100 nM Dex in the absence or presence of ZM 182780 markedly blocked DNA fragmentation induced by the low doses of H_2O_2.

Characteristics of oxidant-induced death

As loss of $\Delta\Psi_m$ is one of the earliest events in apoptosis, the effect of H_2O_2 in combination with ZM 182780 alone, with Dex and with the antiglucocorticoid, RU 486 was determined as a function of time after treatment. Cells pretreated for 48 h with ZM 182780 (100 nM) alone or in combination with Dex (100 nM) and RU 486 (10 μM) were exposed to 150 μM H_2O_2 for various times over a 4- to 5-h period, prior to the addition of a mixture of the $\Delta\Psi_m$-sensitive fluorochrome, $DiOC_6$, and the marker for membrane integrity, PI, as described in Materials and Methods. For all experiments conducted ($n=3$), it was observed that after 90-min exposure to H_2O_2 the $\Delta\Psi_m$ decreased for cells pre-exposed to ZM 182780 and this was paralleled by the uptake of PI. Figure 5 shows the results from one experiment typical of all three. It is clear that in the presence of ZM 182780, a second lower intensity peak was observed for $DiOC_6$ and this correlated with a large uptake of PI as indicated by the adjacent profiles. Both the lower intensity fluorescence peak, corresponding to cells with a low $\Delta\Psi_m$, and the high intensity red fluorescence peak, indicating PI uptake, were markedly attenuated by Dex; a response reversed by RU 486. It should be noted that, in this experiment, no marked effect on $\Delta\Psi_m$ and PI uptake could be observed when H_2O_2 was added alone to cells (control). Although only the 90-min exposure time is shown in Fig. 5, for all experiments conducted, Dex was still able to significantly reduce H_2O_2-induced PI uptake after exposure to H_2O_2 for 4 h (ZM 182780+H_2O_2 vs ZM 182780+Dex+H_2O_2; 67 ± 12% vs 26 ± 8%, $P<0{\cdot}01$; paired *t*-test at 90 min, 1, 2, 3 and 4 h for data from three experiments).

For all three experiments, the percentage of the cell population with a low $\Delta\Psi_m$ was correlated with the proportion of the cell population showing PI uptake, for the 90 min, 2, 3 and 4 h sampling times (Fig. 6). Statistical

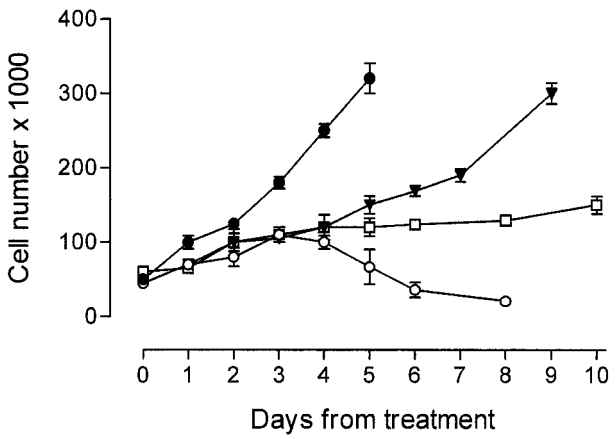

Figure 3 Growth curves for cells treated with ZM 182780 (100 nM) alone (○), for cells treated with Dex (100 nM) alone (▼), and for ZM 182780 and Dex (□), over an extended time-course of 10 days. Control (●).

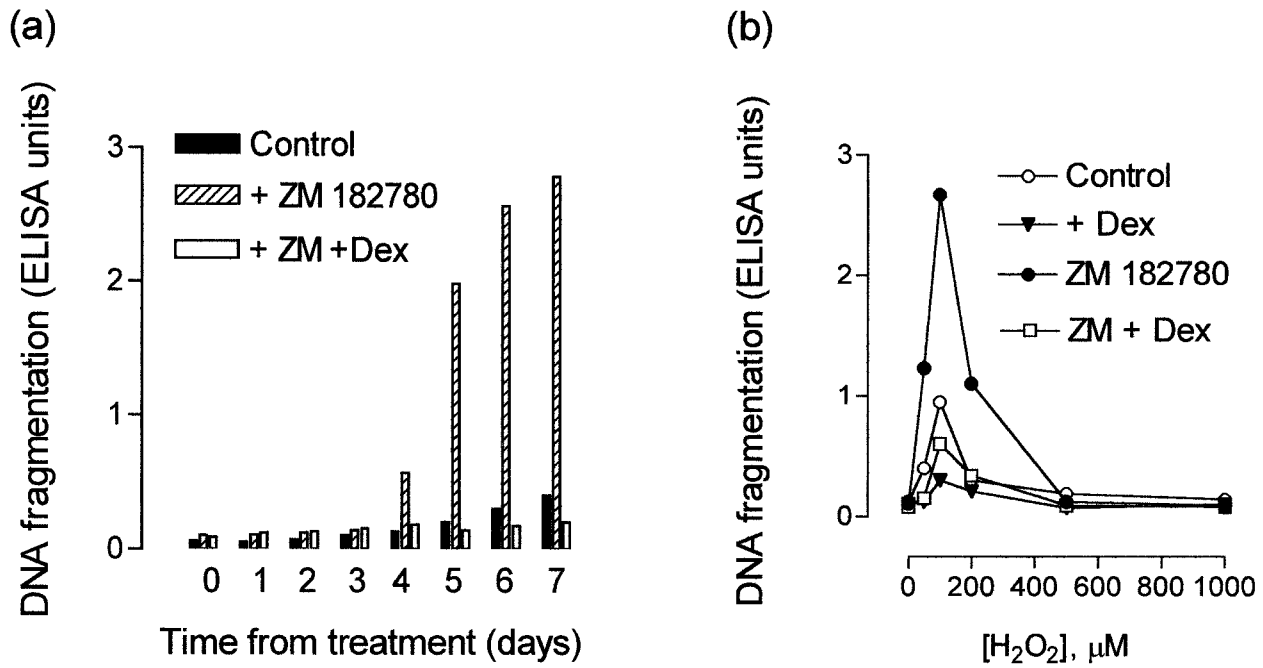

Figure 4 The ability of the glucocorticoid, Dex, to block antioestrogen-induced and exogenous oxidant-induced cell death. In (a) the time-course for the response to ZM 182780 (ZM; 100 nM) and Dex (100 nM) is presented, whilst in (b) the dose-response to a 2-h exposure to H_2O_2 in the presence of ZM 182780 (100 nM), and/or Dex (100 nM) is shown.

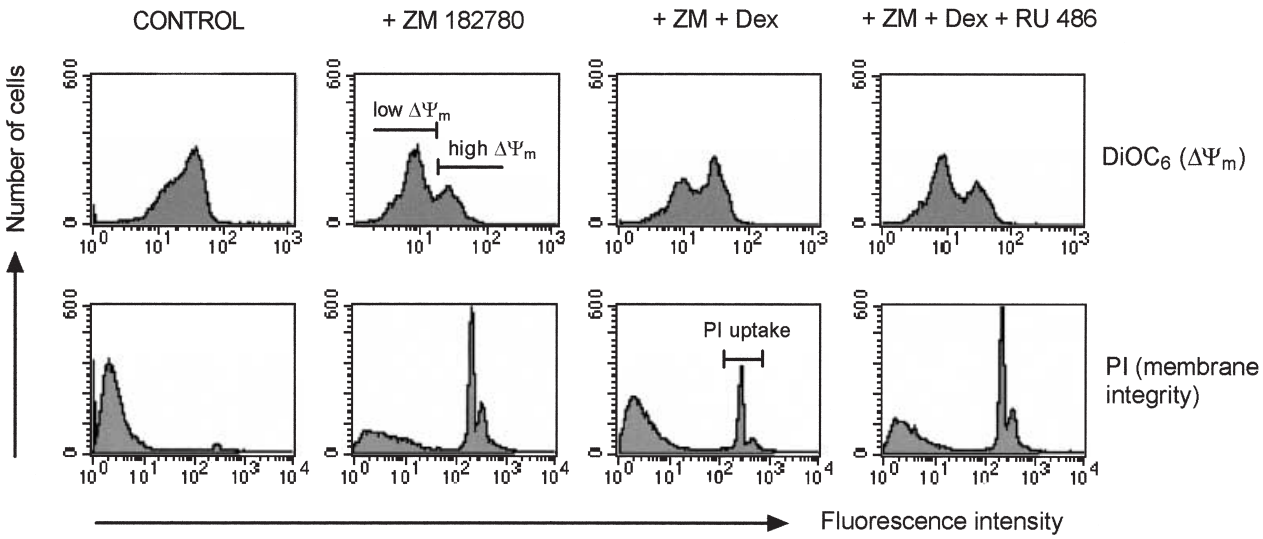

Figure 5 $\Delta\Psi_m$ and cell membrane integrity in response to 90-min exposure to H_2O_2 following pre-treatment (48 h) of cells with ZM 182780 (ZM; 100 nM), or with ZM 182780 and Dex (100 nM) or ZM 182780, Dex and the antiglucocorticoid, RU 486 (10 μM). The top row of histograms show green fluorescence intensity ($\Delta\Psi_m$) for cells stained with the mitochondrial fluorochrome, $DiOC_6$. The corresponding panels below show the uptake of PI (membrane integrity). Cells with a loss of $\Delta\Psi_m$ are those giving the second low intensity fluorescence peak, whilst loss of membrane integrity is apparent by the appearance of peaks at high red fluorescence.

analysis reveals a correlation coefficient of 0·914 and a slope of 1·07. This analysis shows that at no time-point following H_2O_2 exposure, could the loss of the $\Delta\Psi_m$ be disassociated from the uptake of PI.

A further possibility that might explain the above findings is that membrane integrity is lost following a catastrophic loss of mitochondrial function. To address this, GH_3 cells were stained with the mitochondrial

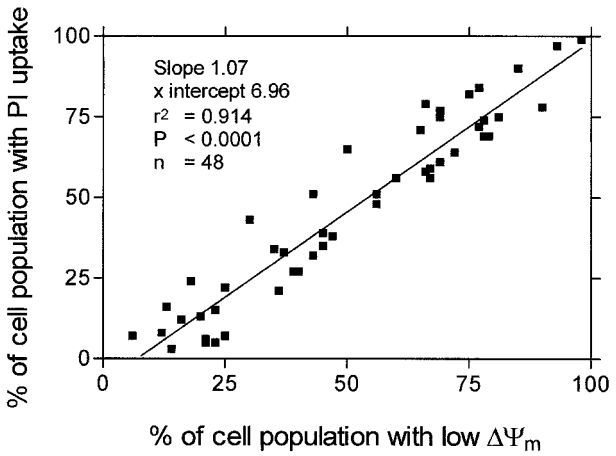

Figure 6 Correlation between the percentage of the cell population with a low $\Delta\Psi_m$ and the percentage that have taken-up PI following treatment with H_2O_2 (150 μM) in combination with ZM 182780 (100 nM), Dex (100 nM) and RU 486 (10 μM). Values shown were obtained after 90 min, 2 h, 3 h and 4 h exposure to H_2O_2 and were from three different experiments.

fluorchrome, JC-1. Like $DiOC_6$, this cationic compound accumulates in mitochondria but, unlike $DiOC_6$, it has the further property that at high $\Delta\Psi_m$, it forms red fluorescent 'J' aggregates. Figure 7a shows cells treated with JC-1 alone for 30 min (positive control for $\Delta\Psi_m$) in comparison with cells treated with JC-1 and the protonophore, mClCCP (100μM; Fig. 7b) where orange/red fluorescence is absent (negative control for $\Delta\Psi_m$). Figure 7c–h shows the results of another experiment where in comparison with the control for the addition of JC-1 (Fig. 7c) and Hoechst 33342 alone (Fig. 7d), around 50% of cells have a low $\Delta\Psi_m$ (Fig. 7e) and a similar proportion have lost membrane integrity (Fig. 7f), as indicated by the nuclear uptake of PI (red nuclei), when treated for 90 min with 150 μM H_2O_2. In contrast, whilst all cells treated with 1 mM H_2O_2 for 90 min have a low $\Delta\Psi_m$ (Fig. 7 g), none of these cells show PI uptake (Fig. 7 h). Although not shown, these cells treated with the high dose of H_2O_2 continued to exclude PI for 4–5 h. These data strongly suggest that loss of $\Delta\Psi_m$ is due to a catastrophic loss of cell membrane integrity and not vice versa.

Mechanism for Dex blockade of antioestrogen- and oxidant-induced death

Cellular ATP concentrations in response to H_2O_2 Cell death involving a loss of membrane integrity is often associated with insufficient cellular ATP to maintain plasma membrane Na^+/K^+-ATPase activity. Experiments were conducted to determine the ATP status of cells in relation to the time at which PI uptake occurred following challenge with H_2O_2. Figure 8a shows that after 30-min exposure of cells to 1 mM H_2O_2, ATP concentrations had fallen to 10% of control values. At this time-point, Fig. 8b shows that PI uptake had not occurred. After a further 30 min, Fig. 8a shows that at the low pro-oxidant concentration of 150 μM, ATP concentrations continued to fall and that this was followed by the uptake of PI, indicating a loss of cell membrane integrity. That ATP levels had fallen so dramatically at the high H_2O_2 doses and yet cell death (loss of membrane integrity) failed to occur for several hours, suggested that it was not the absolute cellular ATP concentration that was the factor determining cell survival.

Effect of inhibiting glycolysis and mitochondrial function on response to H_2O_2 A further finding was that at the low oxidant doses cells displayed marked membrane blebbing. Figure 9 shows that in comparison with cells treated with a low dose of H_2O_2 (Fig. 9b), for high pro-oxidant doses (Fig. 9c), or where cells were pretreated with ZM 182780 (Fig. 9d), membrane blebbing failed to occur. Membrane blebbing is a process of cytoskeletal arrangement and it can precede apoptosis and necrosis (Schwartzman & Cidlowski 1993). Studies have indicated that glycolytic enzymes are associated with actin and also tubulin cytoskeletal filaments and that glycolysis provides the ATP necessary for the cytoskeletal changes involved in membrane blebbing (Glass-Marmor & Beitner 1997).

To determine the relative role of glycolytic and mitochondrial ATP generation in the response to H_2O_2, cells were incubated in medium lacking glucose or with the protonophore, carbonyl cyanide m-chlorophenylhydrazone (mClCCP) (100 μM; Sigma) and the succinate dehydrogenase inhibitor, 3-nitropropionic acid (3NP; 2 mM). As indicated in Fig. 7b the protonophore collapses the $\Delta\Psi_m$ whilst 3NP will reduce the delivery of electrons to the electron transport chain and lower the $\Delta\Psi_m$ (Keller *et al.* 1998). Figure 10 shows that the removal of glucose markedly enhanced cell death induced by H_2O_2. Surprisingly, both mClCCP and 3NP gave some degree of protection against the oxidant-induced cell death.

Role of GADPH in the response of cells to antioestrogen and Dex As the experiments above strongly pointed to a fundamental role of glycolysis in the response of these cells to oxidants, experiments were performed to determine the extent to which ZM 182780 and Dex exposure altered GAPDH activity, a rate-limiting enzyme of glycolysis (Sahlin 1995). For cells treated with ZM 182780 and Dex for 48 h, enzyme activity was measured in cell lysates. Table 1 shows that ZM 182780 and Dex treatment alone had no effect on enzyme activity. This experiment was then repeated where cells exposed to ZM 182780 and Dex for 48 h were exposed to 150 μM H_2O_2,

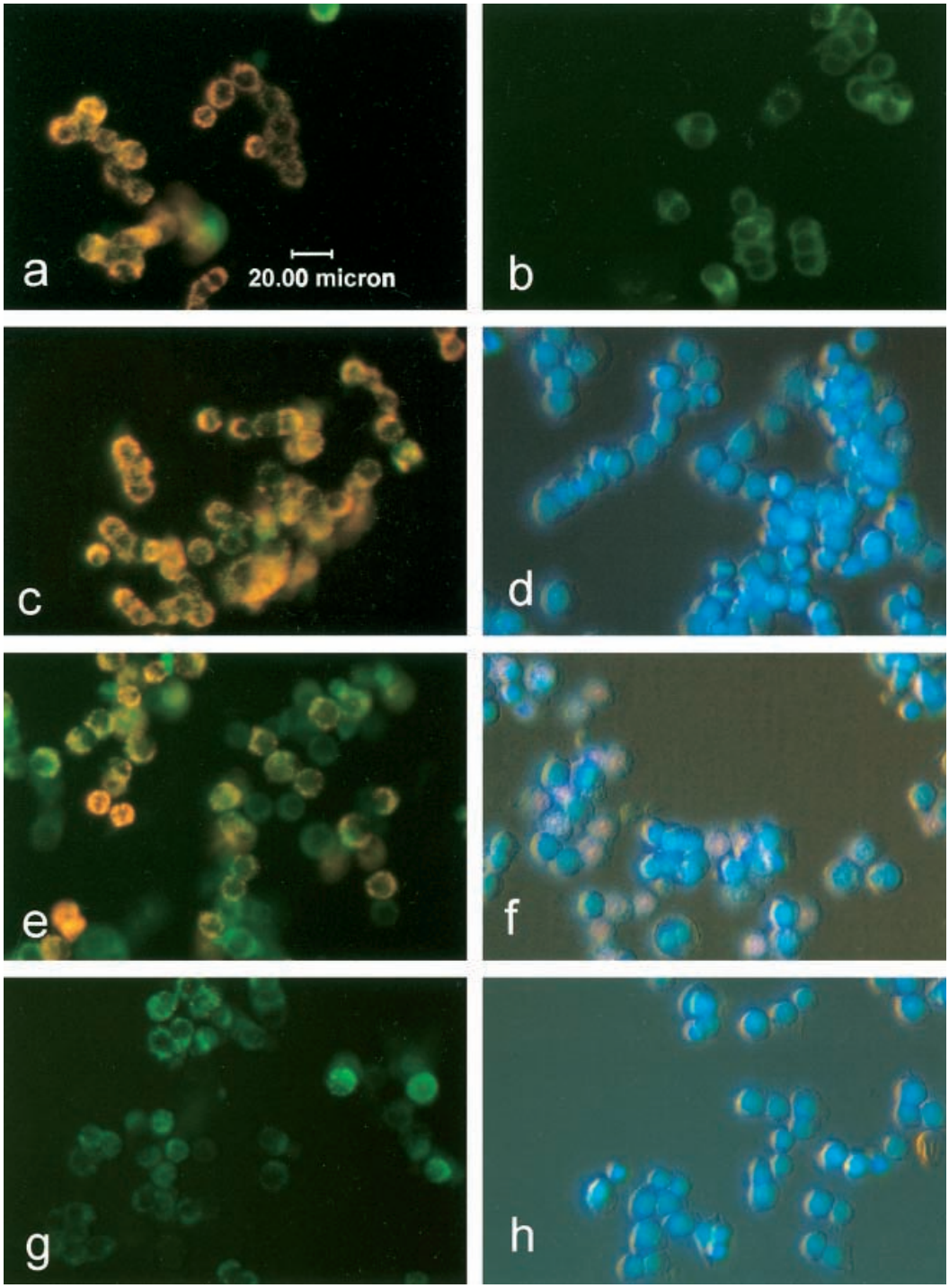

Figure 7 Fluorescence microscopy for $\Delta\Psi_m$ with JC-1 (a, b, c, e, g), and for membrane integrity with Hoechst 33342 and PI (d, f, h). In comparison with cells treated with JC-1 alone (a) where red 'J' aggregates are present (high $\Delta\Psi_m$) (b) shows only green fluorescence (low $\Delta\Psi_m$) in the presence of the protonophore, mClCCP (100 μM). In a separate experiment, (c–h) show the response to treatment with H_2O_2 for 90 min. Control cells with JC-1 and Hoechst 33342 are shown in (c) and (d) respectively, whilst (e) and (f) show the effect of low concentrations of H_2O_2 (150 μM) with JC-1 and Hoechst 33342 respectively. (The same field for JC-1 and Hoechst 33342 could not be used as JC-1 and Hoechst 33342 gave mutual quenching.) Finally (g) and (h) show the response to the high concentration of H_2O_2 (1 mM) with JC-1 and Hoechst 33342 respectively.

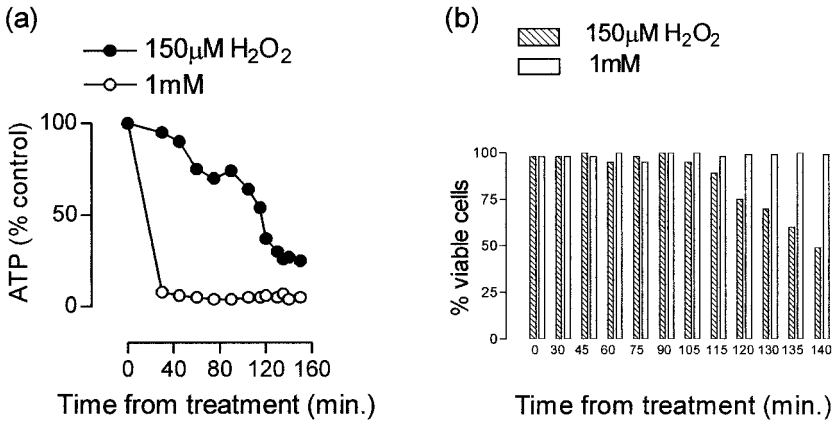

Figure 8 ATP concentrations (a) and cell membrane integrity (b) measured at various times after the addition of H_2O_2 (150 μM). Values are expressed as per cent control.

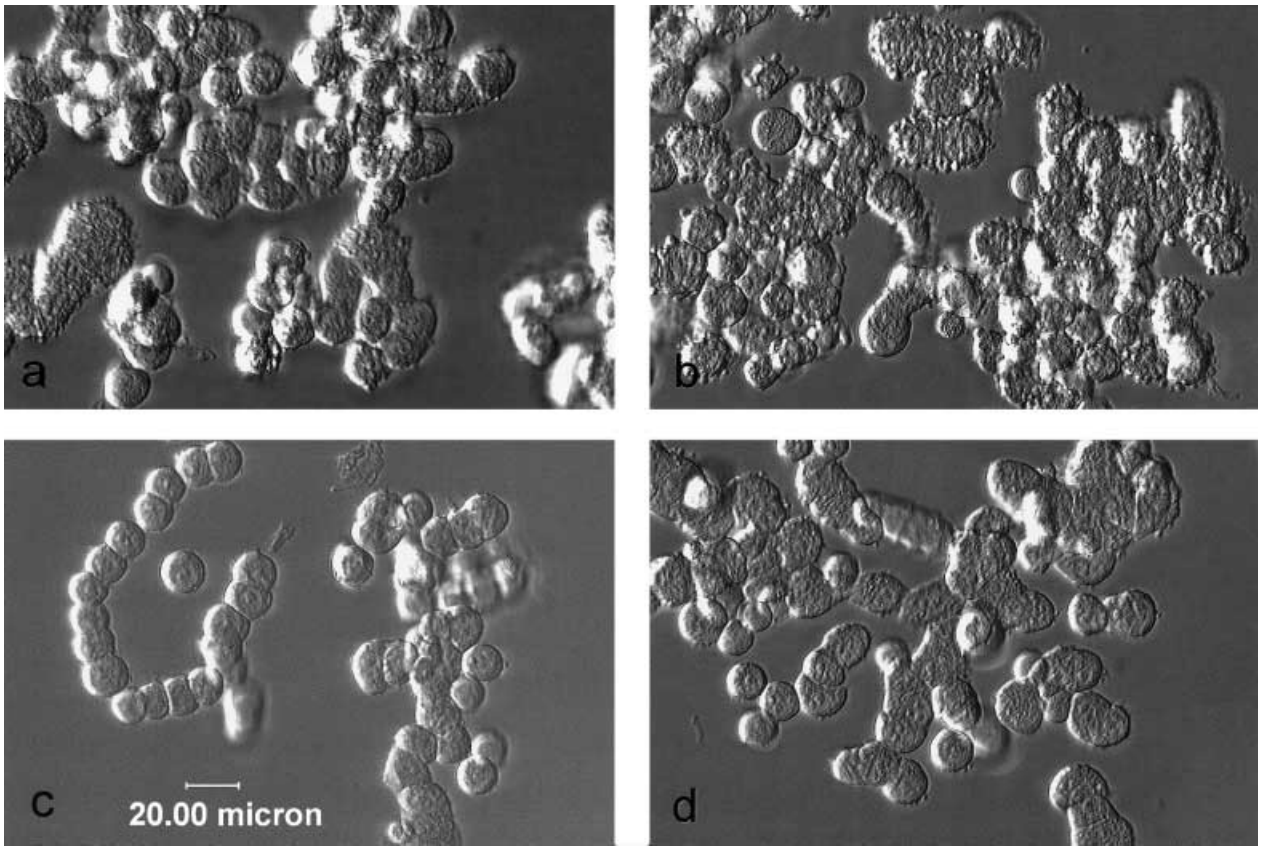

Figure 9 Image Modulated Contrast light microscopy images of GH_3 cells showing membrane blebbing on exposure to 150 μM H_2O_2 for 45 min (b), in comparison with lack of blebbing for cells treated with 1 mM H_2O_2 (c) or for cells treated with 150 μM H_2O_2, following pre-exposure to ZM 182780 (d). Control, untreated cells are shown in (a).

30 min prior to harvesting for enzyme measurement. As observed in the first experiment, antioestrogen or Dex treatment alone failed to provoke a response but, this time, a fall in enzyme activity occurred for cells exposed to H_2O_2 for 30 min. This decrease of enzyme activity was substantially enhanced in the presence of ZM 182780 and this enhancement was reversed by the inclusion of Dex in the culture medium.

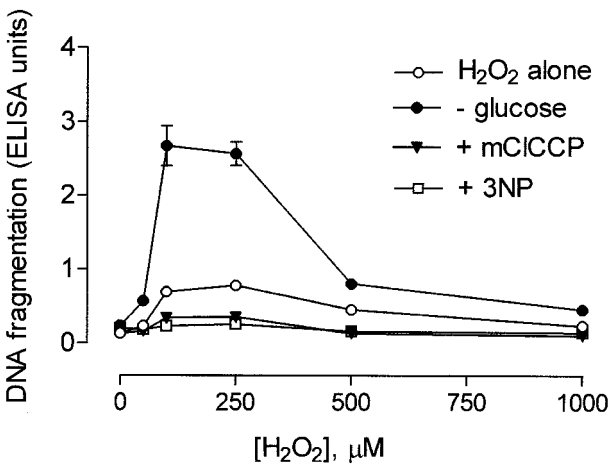

Figure 10 Cell death, as measured by the DNA fragmentation ELISA, in response to H_2O_2 (150 μM) for cells maintained in the absence of glucose or presence of the protonophore, mClCCP (100 μM) or the succinate dehydrogenase inhibitor, 3NP (2 mM).

Table 1 GAPDH activity detected in GH_3 cells exposed to ZM 182780 (100 nM) and Dex (100 nM) in the absence and presence of H_2O_2 (150 μM). Values (means ± S.D.) are given as percentage of control

	Enzyme activity (% control)
Treatment and experiment	
Experiment 1	
Dex	94 ± 8
ZM 182780	89 ± 14
Experiment 2	
Dex	107 ± 10
ZM 182780	94 ± 8
H_2O_2 (150 μM)	56 ± 12[a]
H_2O_2+ZM 182780	23 ± 8[b]
H_2O_2+ZM 182780+Dex	64 ± 7[c]

[a]P<0·001 vs control; [b]P<0·01 vs H_2O_2 alone; [c]P<0·01 vs H_2O_2+ZM 182780.

Discussion

The expansion of a cell mass is dependent on stimuli that activate cell cycle events and those that allow the survival of cell progeny. With regard to the growth and development of rat pituitary tumours, oestrogens are the endocrine agents that would appear to fulfil both these requirements. Therefore, the rat pituitary tumour cells used here are not only dependent on the ER for growth but, in the presence of the pure antioestrogens, ZM 182780 and RU 58668 (Van de Velde *et al.* 1994), they are killed. During studies to determine the role of other steroid-activated pathways in this response, we have observed that death induced by antioestrogens was blocked by the glucocorticoid, Dex. These observations contribute to the growing controversy concerning the role of glucocorticoids in cell survival.

A classical model of apoptosis is the glucocorticoid-induced death of thymocytes (Schwartzman & Cidlowski 1994). Further support for a pro-death role of glucocorticoids comes from the studies on Alzheimer's disease. Alzheimer's disease is associated with aging and hippocampal cell death and there is an age-related increase in the activity of the hypothalamus–pituitary axis (Behl *et al.* 1997). These observations have prompted studies showing that Dex enhances oxidant-induced hippocampal cell death (Behl *et al.* 1997, Reagan & McEwen 1997). In addition to this, studies have demonstrated that glucocorticoids enhance neuronal ischaemic necrosis (Sapolsky & Pulsinelli 1995). Although necrosis can be due to physical cell injury, in the majority of cases it is due to severely compromised cell energetics (Eguchi *et al.* 1997, 1999). During ischaemic injury, mitochondrial function is restricted by low O_2 concentrations, rendering cells dependent on glycolytic function (Maxwell & Lip 1997). Glucocorticoid-enhanced necrosis under these circumstances may be due to the observed down-regulation of glucose uptake by these hormones (Rist & Naftalin 1991). Contrary to these reports, it has been demonstrated that Dex prevents cell death in a neonatal rat model of hypoxic–ischaemic encephalopathy (Ekert *et al.* 1997). Here it was reported that cell death was by apoptosis. Similarly, there are other reports to indicate that liver cell, neutrophil, pancreatic acinar cell and breast cell apoptosis is blocked by glucocorticoids (Feng *et al.* 1995, Harris *et al.* 1995, Liles *et al.* 1995, Iida *et al.* 1998). From these conflicting studies it would appear that the effect of glucocorticoid exposure is very much cell-type dependent. Our study now shows that for a pituitary tumour cell line, cell death in response to oxidants is also blocked by Dex.

Our earlier reports on these pituitary tumour cells maintained that cell death in response to the antioestrogen ZM 182780 and to exogenous oxidants had more of the characteristic of apoptosis than necrosis (Newton 1995, Newton *et al.* 1999). Having performed more extensive studies on these cells, we now believe that cell death induced by antioestrogens and oxidants is more characteristic of necrosis. The confusion with regard to assigning the correct mode of cell death has arisen for several reasons. The first of these is that antioestrogens cause considerable cell shrinkage prior to rapid death – this is a characteristic of apoptosis in a number of systems (Schwartzman & Cidlowski 1993). We have now performed experiments showing that a reduction in cell volume in response to ZM 182780 is not always followed by cell death (data not shown). Secondly, using an ELISA assay that fails to detect necrosis in endothelial cells (Newton *et al.* 1999), marked DNA fragmentation was detected in response to antioestrogen and oxidants. The third factor that has given rise to this confusion is the unusual dose-response to H_2O_2. In

the majority of cell systems, low oxidant doses induce apoptosis and high doses induce necrosis. For GH_3 cells, we have observed that low doses of H_2O_2 induce rapid death whilst death at higher doses is delayed for several hours.

A re-evaluation of the characteristics of oxidant-induced cell death, as presented here, has provided the findings that the loss of $\Delta\Psi_m$, a characteristic response of early apoptosis in a number of systems, cannot be disassociated from a loss of cell membrane integrity (see Figs 5 and 6). In practical terms, at every time-point of $\Delta\Psi_m$ measurement with the cationic fluorochrome, $DiOC_6$, PI uptake correlates with the fall in $\Delta\Psi_m$. This strongly suggests that a catastrophic loss of membrane function has occurred resulting in the loss of mitochondrial function. Data presented in Fig. 7 showing that at high H_2O_2 doses, cell membrane integrity is intact when the $\Delta\Psi_m$ has collapsed indicate that cell membrane function is not lost as a secondary response to a collapse of mitochondrial function and ATP generation. Indeed data presented in Fig. 8 indicate that the absolute level of ATP, measured in the whole population of cells under treatment, does not determine the viability of the cells. Therefore, high doses of H_2O_2 induce a large fall (95%) in ATP concentrations and yet cell membrane integrity is maintained for several hours. In contrast, at the lower doses of H_2O_2, loss of cell membrane function is apparent when ATP concentrations have only fallen to around 30–40% of control values. Our observations of a rapid uptake of PI indicate a catastrophic loss of membrane function for a proportion of the cell population. One of the possibilities we have considered is that such an effect was due to oxidation of membrane lipids. Although not shown, we have attempted to address this by determining glutathione (GSH) concentration with the fluorescent probe, Cell Tracker Green (Molecular Probes). In our earlier publication we have shown that GSH is a major antioxidant in these cells (Newton *et al.* 1999). In the presence of GSH and the enzyme glutathione-S-transferase, Cell Tracker Green is converted to a green fluorescent compound. Using this method, we have been unable to detect a loss of GSH in response to H_2O_2 until the time at which cell membrane rupture has occurred. For this reason, we believe that the loss of membrane integrity has, paradoxically, more to do with the energetic status of the cells than the antioxidant status. In particular, we propose that high oxidant doses induce a 'shut-down' of cellular activities, sparing the remaining ATP for the maintenance of cell membrane function. At the lower doses of H_2O_2, ATP-consuming cellular processes will not be as markedly inhibited and therefore the demand on the diminishing pool of ATP will be higher. As the cell energetics continue to fall in response to oxidative damage, a crisis point will be reached at which demand outstrips supply, leading to a rapid and catastrophic loss of plasma membrane Na^+/K^+-ATPase and membrane integrity. Our inability to measure this rapid fall in ATP concentrations on an individual cell basis before membrane integrity is lost is due to the current lack of suitable methodology.

With regard to the mechanism by which Dex blocks antioestrogen- and oxidant-induced cell death, we have had to first identify a mechanism by which oxidants induce cell death. Given our observations in Fig. 9 concerning membrane blebbing, in the light of studies indicating that glycolysis drives the cytoskeletal changes that result in blebbing, we have tested the relative role of glycolysis and mitochondria in the response to H_2O_2. Data presented in Fig. 10 have shown that removal of glucose markedly enhances oxidant-induced cell death whilst, paradoxically, lowering mitochondrial function is protective. Previous studies by Sahlin (1995) have demonstrated that a key enzyme of glycolysis, GAPDH, is highly regulated by oestrogens and by Dex. In addition, studies have shown that GAPDH is a major target for oxidative damage due to the presence of a critical thiol group (Baker *et al.* 1989). Our data presented in Table 1 show that whilst enzyme activity is not markedly altered in response to ZM 182780 and Dex alone, in the presence of H_2O_2, antioestrogen pretreatment leads to an enhanced reduction of GAPDH activity following H_2O_2 exposure, and this effect is blocked by Dex. These data strongly indicate that one of the mechanisms by which Dex is able to block antioestrogen-enhanced, oxidant-induced cell death is that the steroid stabilises glycolytic function. In contrast to these results on glycolytic function, the results presented in Fig. 10 have provided the intriguing possibility that mitochondrial function, in part, drives the death process. Here it was shown that the two agents that lower mitochondrial activity reduce death in the presence of the low doses of H_2O_2.

These data presented in the current report support the previous studies on GH_3 cells that antioestrogens enhance sensitivity to the toxic effect of oxidants. We now show that this effect is blocked by the glucocorticoid, Dex, possibly due to the role that the Dex-activated GR has to stimulate glycolysis – a pathway that we show here to be fundamental in the response of these cells to oxidants. These finding therefore contribute to the growing body of data to suggest that, for certain cell types, glucocorticoids decrease susceptibility to toxic insults. Cellular energetics have been shown to be fundamental with regard to how a cell responds to a particular toxic agents. Apoptosis is an energetic process and stimuli that normally induce apoptosis have been shown to induce necrosis when cellular ATP levels are reduced (Eguchi *et al.* 1997, 1999). By blocking necrosis, one might have expected that Dex would allow apoptosis to occur. The failure to observed apoptosis is not due an inability of GH_3 cells to undergo apoptosis, as we have recently observed this mode of death in response to the calmodulin inhibitor, calmidzolium chloride (Newton *et al.* 2000). Our most recent unpublished observations on these cells may provide the explanation for this. In response to the topoisomerase

inhibitor, camphothecin, GH_3 cells undergo nuclear fragmentation characteristic of apoptosis and this is blocked by Dex. Therefore, our studies concur with those of others showing that glucocorticoids block both necrotic and apoptotic cell death. Although we have provided some evidence to support a mechanism by which necrosis is blocked, studies are required to determine at which step in the apoptotic pathway Dex is able to block apoptosis.

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